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# Histone Modifications as a Platform for Cancer Therapy

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**Abstract** Tumorigenesis and metastasis are a progression of events resulting from alterations in the processing of the genetic information. These alterations result from stable genetic changes (mutations) involving tumor suppressor genes and oncogenes (e.g., *ras*, *BRAF*) and potentially reversible epigenetic changes, which are modifications in gene function without a change in the DNA sequence. Mutations of genes coding for proteins that directly or indirectly influence epigenetic processes will alter the cell's gene expression program. Epigenetic mechanisms often altered in cancer cells are DNA methylation and histone modifications (acetylation, methylation, phosphorylation). This article will review the potential of these reversible epigenetic processes as targets for cancer therapies. J. Cell. Biochem. 94: 1088–1102, 2005.
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**Key words:** histone modifications; DNA methylation; chromatin; carcinogenesis

## MUTATIONS, EPIGENETICS, AND CANCER

Carcinogenesis is a progression of events resulting from alterations in the processing of the genetic information. These alterations result from stable genetic changes (mutations) involving tumor suppressor genes, oncogenes (e.g., *ras*, *BRAF*), DNA stability genes, and potentially reversible epigenetic changes, which are modifications in gene function without a change in the DNA sequence [Egger et al., 2004; Hake et al., 2004; Vogelstein and Kinzler, 2004]. A cell's genome is constantly being challenged with mutations that arise spontaneously or through environmental factors. The efficiency of the cell's safeguard systems in detoxification, apoptosis, and DNA repair will decide the extent that mutations accumulate. Mutations that confer a survival advantage in the cell's environment will put it on the path of tumorigenesis [Ilyas et al., 1999]. Mutations of genes coding for proteins that directly or indirectly influence epigenetic processes will alter genetic programs. Epigenetic mechanisms often altered in cancer cells are DNA methylation and histone modifications. This review will focus on how genetic changes influence epigenetic processes with an emphasis on histone modifications. Further we will discuss how the potentially reversible epigenetic processes are targets for cancer therapies.

## HISTONE MODIFICATIONS AND CHROMATIN REMODELING

Nuclear DNA is packaged into nucleosomes, which consist of a histone octamer core, arranged as a $(H3–H4)_2$ tetramer and two H2A–H2B dimers, around which DNA is wrapped [Davey et al., 2002]. The core histones have a similar structure with a basic N-terminal tail, a globular domain organized by the histone fold, and a C-terminal tail [Luger et al., 1997]. The core histones are reversibly modified by acetylation, methylation, ubiquitination, biotinylation, and phosphorylation [Spotswood and Turner, 2002; Camporeale et al., 2004; Davie, 2004; Peterson and Laniel, 2004]. Until recently it was thought that modifications occurred solely on the N- and C-terminal tails of the core histones. However, analyses of histone modifications by mass spectrometry have revealed several modifications (acetylation and methylation) in the histone fold [Zhang et al., 2003; Freitas et al., 2004] (Fig. 1).

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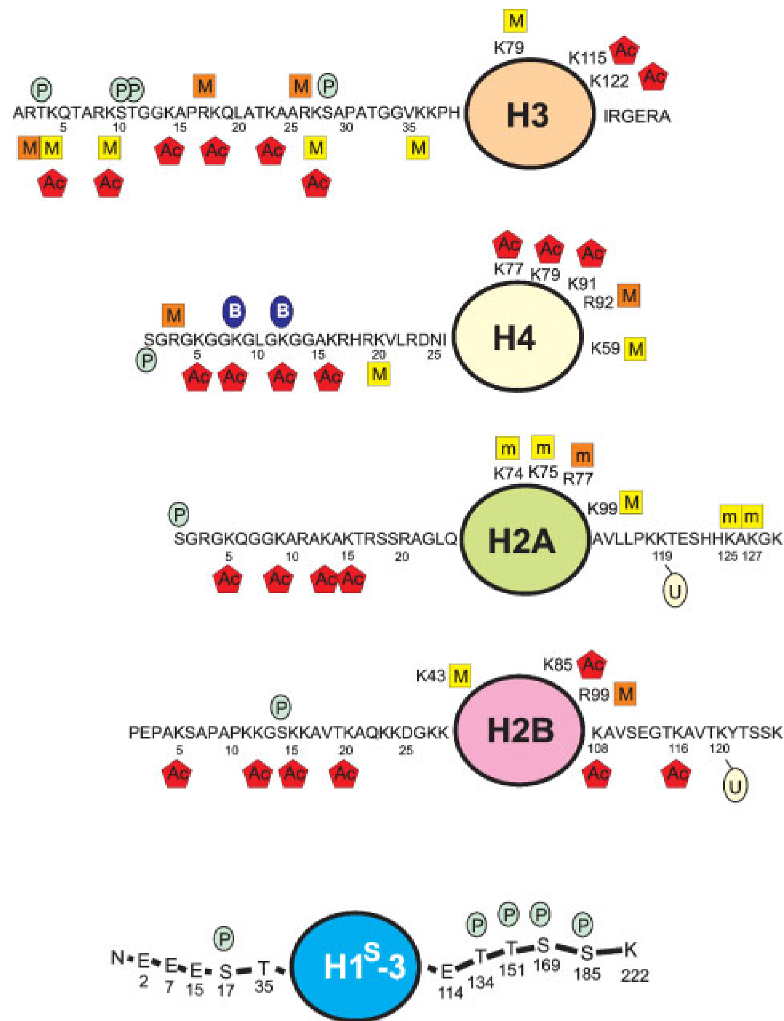


**Fig. 1.** Core and H1 histone modifications. Human core histone amino acid sequences are shown. Histone H1$^{S}$-3 is a mouse H1 subtype. The modifications include methylation (M), acetylation (Ac), phosphorylation (P), ubiquitination (U), and biotinylation (B). Methylation sites that are uncertain are denoted as (m). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

At physiological ionic strength chromatin is folded into higher order structures that are stabilized by core histone N-terminal tails and H1, which binds to the linker DNA that joins nucleosomes together [Van Holde and Zlatanova, 1996]. Linker histone H1 is modified by phosphorylation. Modifications of the core histone tails and H1 destabilize higher order chromatin structure. Of the core histones, H4 has a prominent role in the compaction of the chromatin fiber [Dorigo et al., 2003]. The H3 N-terminal tails project further than other core histone tails from the nucleosome [Leuba et al., 1998]. It has been suggested that the modifications occurring on the H3 tail provide regulatory information. Modifications of specific histone residues are required for interactions with specific protein domains [Bottomley, 2004; Hake et al., 2004]. For example, the bromodomain found in transcription factors and chromatin remodeling proteins binds to acetylated lysine residues.

The chromatin immunoprecipitation assay and yeast genetic screens have shown the alignment of specific histone modifications with transcriptionally active or repressed chromatin. Methylated K4 and K79; acetylated K9 and K14 of H3 are associated with transcriptionally active chromatin, while methylated K9 H3 is with repressed chromatin of mammalian cells [Liang et al., 2004; Schubeler et al., 2004]. The activation or repression of mammalian genes involves chromatin remodeling by histone modifying enzymes and ATP-dependent chromatin

remodeling complexes (e.g., SWI/SNF) [Peterson and Laniel, 2004]. Histone acetyltransferases (HATs) and histone deacetylases (HDACs), which catalyze reversible histone acetylation, are among the best understood histone modifying enzymes in terms of multiprotein components, mechanisms of recruitment to regulatory elements of genes and role in transcription. Transcription factors recruit coactivators with HAT activity (e.g., p300/CBP) to regulatory DNA sites, while transcriptional repressors recruit corepressors with HDAC activity [Davie and Moniwa, 2000; Hake et al., 2004; Peterson and Laniel, 2004]. In transcriptionally poised and active chromatin regions histone acetylation is a dynamic process, with the steady state of acetylated histones being decided by the relative activities of the recruited HAT and HDAC complexes [Katan-Khaykovich and Struhl, 2002; Davie, 2003a]. Histone kinases (e.g., mitogen and stress activated kinase (MSK) 1) are recruited to promoters, but how these enzymes are recruited is poorly understood. The ATP-dependent chromatin remodeling complexes move nucleosomes along the DNA allowing transcription factors, histone modifying enzymes, and the transcription initiation factors access to regulatory DNA sequences [Langst and Becker, 2004]. The temporal order by which histone modifying enzymes and ATP-dependent chromatin remodeling complexes are recruited to DNA is promoter dependent [Martens et al., 2003; Vermeulen et al., 2003].

It is interesting to note that several of the modified amino acids in histone fold are involved in interactions with nucleosomal DNA. Mutations of these amino acids in yeast alleviated the need for chromatin remodeling by SWI/SNF and/or HATs. It has been proposed that modification of these key histone residues may enhance nucleosome mobility and nucleosome dynamics (e.g., histone exchange) [Cosgrove et al., 2004].

## RAS-MITOGEN ACTIVATED PROTEIN KINASE (MAPK) SIGNAL TRANDUCTION PATHWAY

Growth factors (epidermal growth factor, EGF) and phorbol esters (12-*O* tetradecanoylphorbol-13-acetate, TPA) transiently activate the Ras-MAPK pathway (Ras-Raf-MEK-ERK) [Kolch, 2000; Hilger et al., 2002] (Fig. 2). The Ras family members consisting of H-Ras, K-Ras, and N-Ras are proto-oncogenes that are activated by the exchange of GDP with GTP. GTP-bound Ras will activate one of the three Raf proto-oncogene family members (Raf-1, A-Raf, B-Raf), which then results in the activation of a series of kinases, MEKs and ERKs. The amplitude and duration of ERK phosphorylation in response to EGF or TPA varies with cellular backgrounds. For example, ERK activation in mouse 10T½ mouse fibroblasts is maximal at 30 min and then subsides, while in HeLa cells the duration of TPA-induced ERK phosphorylation is shorter than that of the mouse fibroblasts, reaching a maximum at 15 min and then dropping off sharply at 60 min [Allan et al., 2003].

The Ras-MAPK pathway is often deregulated in cancer cells, resulting in constitutive activation of the pathway. Approximately, 30% of human cancers have mutations in *ras* family members. Mutation in codon 12 or 13 results in a GTP bound state of Ras that is constitutively active. Some human cancers have a very high frequency of *ras* mutations, while at other cancer sites *ras* mutations are not common. Mutated K-*ras* is frequently found in colorectal tumors (50%) and pancreatic carcinomas (90%). Further, *BRAF* mutations are also frequently observed in different cancers [Davies et al., 2002]. The Ras-MAPK pathway may also be constitutively activated in cancer cells by defective or over-expressed cell surface receptors (e.g., EGF receptors and HER-2/*neu*/erbB-2 receptors) [Dunn et al., 2005]. Treatment of these cancers with drugs that inhibit Ras, Raf, or MEK have had variable success [Hilger et al., 2002].

In breast cancer, the role of the estrogens and estrogen receptors in hormone dependent progression has been well established. It remains to be evaluated whether the development of endocrine resistance in these neoplasms can be credited to the upregulation of growth factor production from competitive signaling cascades such as the Ras-MAPK pathway. Many signaling molecules that converge upon the Ras-MAPK pathway are overexpressed or amplified in breast cancer. Members of the EGF receptors, particularly Her2/neu or ErbB2 which is overexpressed in 30% of breast tumors, and the insulin receptors/insulin-like growth factor receptors have been implicated in breast cancer proliferation and tumorigenesis [Harari and Yarden, 2000; Surmacz, 2000; Dunn et al., 2005]. Monoclonal antibodies such as Herceptin that block Her2 have shown clinical promise in targeting and treating a subset of patients

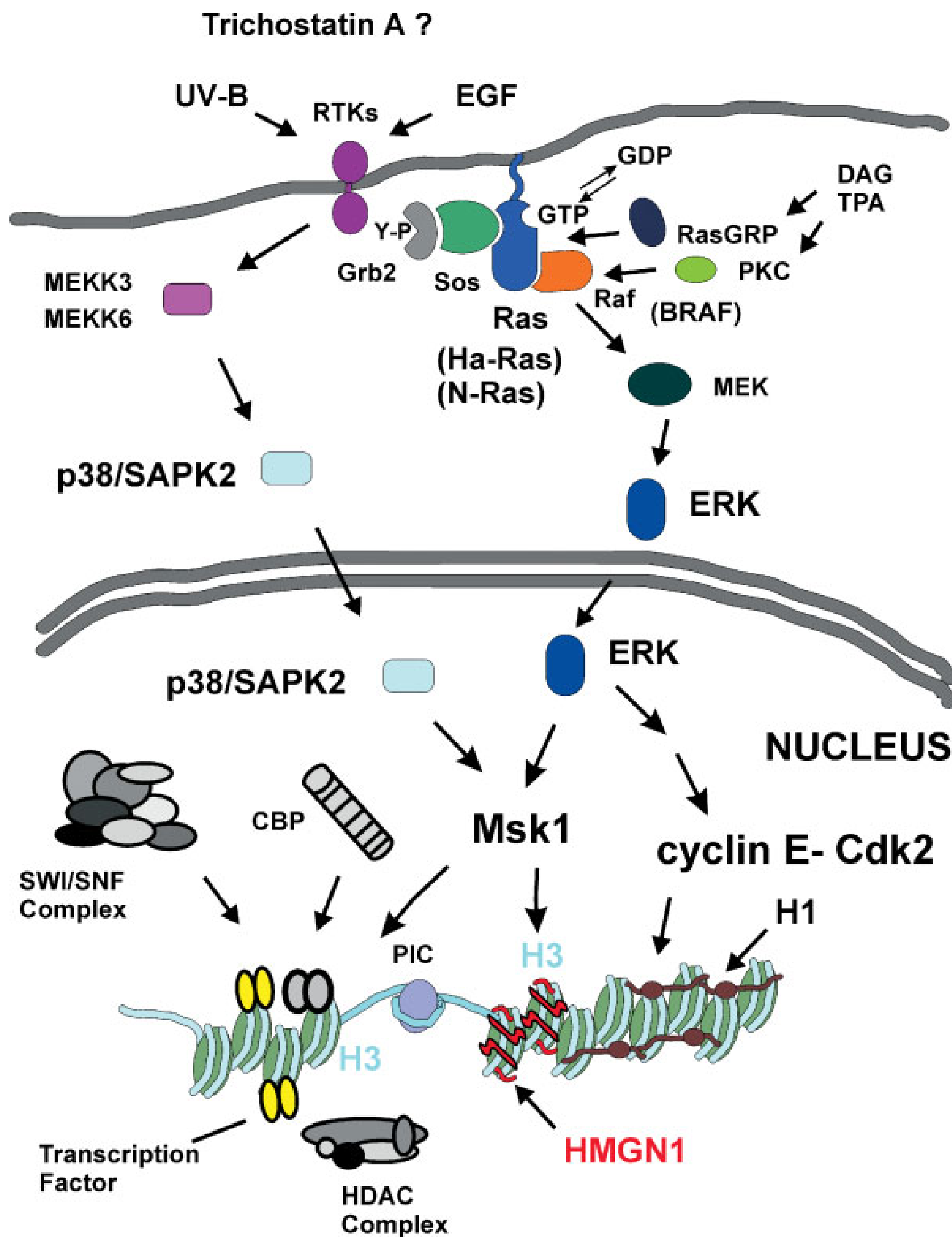


**Fig. 2.** MAPK signal transduction pathways and the modification of chromatin. The Ras-MAPK pathway is activated by EGF and TPA. TPA acts through PKC and/or RasGRP. UV-B activates both the Ras-MAPK and the p38 kinase pathways. RTKs, receptor tyrosine kinases; RasGRP, Ras guanyl nucleotide-releasing protein; DAG, diacylglycerol; PIC, preinitiation complex. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

although poor prognosis and chemoresistance persist in some. The non-receptor tyrosine kinase, c-Src, and adaptor molecule Grb2 that both occur upstream of the Ras-MAPK pathway have also been observed overexpressed and upregulated in breast cancer tumors and cell lines [Biscardi et al., 2000; Malaney and Daly, 2001]. Furthermore, the dually phosphorylated ERK 1 and 2 have been the factors most prominently demonstrated to be linked to estradiol hypersensitivity and the development of hormone independence [Dunn et al., 2005]. These downstream kinases can mediate phosphorylation of estrogen receptor $\alpha$ at S118 that enhances the transcriptional activity of the receptor and permit ligand-independent activation [Kato et al., 1995]. With the extensive involvement of factors associated in Ras-MAPK

signaling in breast cancer, it is evident that there are multiple avenues from which interactions and synergistic relationships between growth factor receptors with hormone receptors can arise adding a level of complexity to the development of the disease as well as possible paradigms of breast cancer progression.

## RAS-MAPK, H3 PHOSPHORYLATION, AND CHROMATIN

Stimulation of the Ras-MAPK pathway results in the activation of a series of kinases and transcription factors, the modification of chromatin proteins, and the activation of genetic programs [Hazzalin and Mahadevan, 2002]. TPA or EGF stimulation of mouse fibroblasts, for example, results in the phosphorylation of H3 at S10 and S28 and HMGN1 at S6 [Strelkov and Davie, 2002; Soloaga et al., 2003; Lim et al., 2004]. The amplitude and duration of TPA-induced phosphorylation of H3 at S10 and S28 parallels that of the activated phosphorylated ERKs. Treatment of cells with MEK inhibitors before EGF or TPA stimulation blocks these phosphorylation events [Mahadevan et al., 1991; Barratt et al., 1994; Chadee et al., 1999; Clayton and Mahadevan, 2003; Lim et al., 2004]. Defined temporal patterns and induction levels of immediate early genes such as c-*fos* and c-*jun* are dependent on the specific stimuli applied and the cellular context. Others and we have used the chromatin immunoprecipitation assay to demonstrate that TPA/EGF-induced phosphorylated S10 H3 is associated with the promoter and coding regions of immediate early genes (c-*jun*, c-*fos*, and c-*myc*) in mouse fibroblasts [Chadee et al., 1999; Cheung et al., 2000; Clayton et al., 2000; Thomson et al., 2001]. Inhibition of MEK activity with PD98059 or UO126 prevented TPA induction of these genes. Different stimuli have characteristic routes in triggering a common nucleosomal response like histone and HMG protein modifications. For example, Mahadevan and colleagues demonstrated that while TPA stimulation mediates its nucleosomal response of H3 and HMGN1 phosphorylation through an ERK-dependent cascade, anisomycin treatments proceed via p38 MAPK pathway [Thomson et al., 1999]. Further, the same group showed that arsenite elicits both H4 acetylation and H3 phosphorylation on *Hsp70* gene through the p38 pathway whereas heat shock prompts H4 acetylation independent of p38 signaling on the same gene [Thomson et al., 2004]. These studies demonstrate that depending on the stimuli, multiple pathways can be activated leading to chromatin remodeling and transcriptional activation of a specific gene.

Constitutive activation of the Ras-MAPK pathway by oncoproteins results in increased steady state levels of phosphorylated H3. Further, the level of phosphorylated $H1^s$-3 is elevated in the oncogene-transformed mouse fibroblasts [Chadee et al., 1995, 1999, 2002]. $H1^s$-3 phosphorylation is unique among the histone modifications in that it requires ongoing transcription or replication for phosphorylation to occur. This observation suggests that chromatin disruption or remodeling during transcription or replication is required for this H1 subtype to be exposed to the H1 kinase, cyclin E-cyclin dependent kinase (Cdk) 2. The increased phosphorylation of these histones, which have key roles in chromatin condensation, are likely responsible for the less condensed chromatin structure of the *ras*-transformed mammalian cells relative to parental cells [Chadee et al., 1995]. *Rb*-deficient human fibroblasts also have increased levels of phosphorylated H1 and a relaxed chromatin structure [Herrera et al., 1996]. Thus, mutations in proto-oncogenes or deletions of tumor suppressor genes influence epigenetic programs altering chromatin structure and function.

Investigators commonly use pharmacological inhibitors that selectively or preferentially interfere with kinases to determine which signaling cascades are involved. The caveat is that the inhibitory effects of these compounds likely prevent more than one kinase aside from the preferred substrate and therefore systematic in vivo evaluation of their actions must be assessed. H89, a member of H7 series inhibitors, preferentially targets MSK1 and 2 at 10 μM but is equally potent against PKA, S6K1, and ROCK-II [Thomson et al., 1999; Davies et al., 2000]. The use of the H89 inhibitor has been paramount in critically assessing the role of MSK1/2 in nucleosomal responses such as H3 and HMGN1 phosphorylation complementing recent knock-out studies [Soloaga et al., 2003], as well as the effect of diverse stimuli on immediate early gene expression [Thomson et al., 1999; Strelkov and Davie, 2002]. In wild-type or parental mouse fibroblasts, immediate early gene induction in the presence of H89 shows

slight reduction and even delayed expression. However, H89 does not influence activation of upstream effector kinases or subsequent transcription factor activation (e.g., ATF2, c-Jun, CREB) [Thomson et al., 1999; Strelkov and Davie, 2002]. Inhibition of immediate early genes by H89 appears more dramatic and acute in *ras*-transformed Ciras-3 cells that inherently have constitutively activated signaling [Strelkov and Davie, 2002]. A possible scenario to explain this observation is that the balance and complexity of players (e.g., changes in expression of transcription factors or activities of chromatin modifying activities) in these cells have shifted, resulting in epigenetic processes that are different from that of the parental cells. Progression to a "cancer" phenotype typically entails upregulation, overexpression, or constitutive activation of proteins/enzymes involved in or regulating epigenetic processes. These changes in the regulation and activity of the epigenetic processes may make the cancer cell more reactive to specific kinase inhibitors, providing the basis for the heightened sensitivity of the *ras*-transformed Ciras-3 cells to H89.

## HISTONE KINASES AND TRANSFORMED CELLS

During mitosis and meiosis, phosphorylation of H3 at S10 and S28 occurs. These phosphorylation events ensure proper chromosome condensation and segregation [Drobic et al., 2005]. The major kinase responsible for H3 phosphorylation during mitosis and meiosis is Aurora B. Over expression of Aurora B has been observed in many cancer cell lines [Ota et al., 2002]. Further, over expression of Aurora B leads to increased phosphorylation of H3 at S10 and this occurrence is associated with chromosome instability often seen in malignant cells [Katayama et al., 2003]. Recently, a potent and selective Aurora kinase inhibitor VX-680 has been shown to decrease H3 phosphorylation at S10 in MCF-7 cells and inhibit tumor growth in vivo leading to regression of leukemia, colon, and pancreatic tumors. Since VX-680 exerts its effects in various types of cancers it could offer a new approach for the treatment of multiple malignancies [Harrington et al., 2004].

It has been demonstrated that phosphorylated H3 at S10 is also involved in transcriptional activation of genes [Chadee et al., 1999; Soloaga et al., 2003]. The kinases responsible for phosphorylating H3 during this event are MSK1 and 2. These AGC kinase family members are activated either by the Ras-MAPK pathway through direct phosphorylation by MAPKs (ERK1/2) or the p38 stress kinase signaling pathway via p38 (SAPK2) phosphorylation, consequently leading to H3 phosphorylation at S10 and S28 [Davie, 2003b]. Recently, it has been shown that another AGC kinase family member, RSK2, is able to phosphorylate H3 in an in vitro kinase assay [Lim et al., 2004], suggesting that RSK2 could be another H3 kinase. However, we found that RSK2 phosphorylated H2B in vitro and not H3 [Strelkov and Davie, 2002]. Furthermore, in Coffin–Lowry cells that have defective RSK2, the H3 phosphorylation mitogen response was normal. The efficiency of RSKs and MSKs as in vitro H3 kinases was assessed through inhibitor studies. These studies showed that MSKs were responsible for mitogen-induced H3 phosphorylation [Soloaga et al., 2003]. In addition, more convincing evidence supporting MSKs as being the H3 kinases comes from the observation that there is a severe reduction of H3 phosphorylation at S10 and S28 in mouse embryonic fibroblasts from MSK1/2 single and double knockouts. Further, the expression of immediate early genes such as *c-fos* and *c-jun* in the MSK knockouts is reduced suggesting that MSK activity towards H3 is an important event in proper immediate early gene expression [Soloaga et al., 2003]. In terms of oncogenesis, it has been demonstrated that H3 phosphorylation at S10 is elevated in *ras*-transformed cells [Chadee et al., 1999]. We recently showed that this increased phosphorylation in oncogene-transformed cells is due to deregulated Ras-MAPK signaling leading to increased activity of MSK1, which was potently inhibited by H89 [Drobic et al., 2004]. In various human cancers mutated *ras* or aberrant Ras proteins have been observed, creating a possible platform for transduction of deregulated signals to MSKs and other pathway-activated downstream targets collectively inducing aberrant gene expression [Drobic et al., 2005]. Further studies assessing the role of MSKs in cancers known to have constitutively active Ras signaling could elucidate a possible therapeutic approach.

As previously mentioned, MSK knockouts show severe reduction in H3 phosphorylation and a reduced induction of immediate early genes. However, MSKs are known to phosphorylate a range of other substrates including a

**TABLE I. Mitogen and Stress-Activated Protein Kinase (MSK) Substrate Specificity**

| MSK substrate | Targeted sequence motif (**RXS**) |
|---|---|
| Histone H3 | ....QTA**RKS**$^{10}$TGG......AA**RKS**$^{28}$AP.... |
| HMGN1 | ...R**KVS**$^{6}$S........PK**RRS**$^{20}$A**RLS**$^{24}$AK... |
| CREB | .....R**RPS**$^{133}$YR.... |
| ATF-1 | .....R**RPS**$^{63}$YR.... |
| p65-NF-κB | .....R**RPS**$^{276}$DRE.... |

nucleosome binding protein HMGN1 and transcription factors such as CREB, ATF-1, and p65 subunit of NF-κB [Dunn et al., 2005]. As seen from Table I, MSKs seem to prefer an RXS motif that most MSK targets contain. Since MSKs target histones, non-histone chromosomal proteins, and transcription factors, it is possible that activated MSKs modulate the transcriptional activity of genes at many different levels. H3 phosphorylation is a rapid process indicating that MSKs would have to phosphorylate nucleosomal H3 in a swift fashion. One possible mechanism for achieving fast chromatin remodeling would be through pre-loading of MSKs at the regulatory DNA elements before induction of the signal transduction pathway. We have shown through protein–DNA formaldehyde cross-linking in parental, *ras*-transformed, and MCF-7 breast cancer cells that MSK1 is bound to regions of chromatin before and after induction with TPA. However, the method utilized in the study does not discriminate if MSK1 was bound directly or indirectly to DNA [Dunn et al., 2005]. In TNF treated L929sA cells MSK1 was recruited to the promoter of IL-6 after TNF induction. Further, the treatment of cells with TNF in combination with H89 prevented loading of MSK1 to the IL-6 promoter [Vermeulen et al., 2003]. In this case MSK1 phosphorylated p65 subunit of NF-κB at S276. In an earlier study, IL-6 gene expression in TNF treated L929sA cells required HAT activity via CBP/p300 [Vanden Berghe et al., 1999]. MSK1 is responsible for phosphorylating p65 subunit and this phosphorylation event could affect p65 DNA binding affinity. However, CBP/p300 might recruit MSK1 to the IL-6 promoter where MSK1 would phosphorylate H3 and promote chromatin decondensation. Upon co-transfection of 293 cells with HA-CBP/p300 and Flag-MSK1, an interaction between CBP/p300 and MSK1 is observed [Janknecht, 2003], suggesting that MSK1 might exert its effects through recruitment via HATs. Therefore, activated MSK1 could activate transcription factors (p65 subunit of NF-κB, CREB, or ATF-1) and affect their ability to bind target DNA and/or affect consequent recruitment of cofactors required for gene expression. Such events facilitate chromatin remodeling and promote transcriptional initiation. A recent study has shown that when HMGN1 binds the nucleosomal core particles, it will directly modulate phosphorylation of nucleosomal H3 by hindering the exposure of the H3 N-terminal domain to MSK1. Phosphorylation of HMGN1 (S6, S20, and S24) by MSK1 precedes that of H3 and leads to weakening of HMGN1 binding to chromatin and consequently an increase in H3 phosphorylation by MSK1 [Lim et al., 2004]. Therefore, MSK phosphorylation of HMGN1 and then H3 may promote nucleosome displacement and chromatin remodeling leading to transcriptional elongation necessary for gene expression. Furthermore, H3 phosphorylation brought upon by MSKs may favor interactions with cofactors and/or SWI/SNF remodeling complexes. Upon inspection of *c-fos* and IL-6 promoter regions, AP-1 regulatory sites are present. Since TNF mediated gene expression requires transcription factor binding to AP-1 regulatory elements, it is conceivable that Fos/Jun family members may also mediate MSK recruitment, however this mechanism needs to be investigated.

## HATs AS THERAPEUTIC TARGETS

The covalent addition of acetyl groups on lysine residues of histones and non-histone proteins is dynamically catalyzed by acetyltransferases that work in concert with deacetylases. Among the N-terminal histone tail modifications, acetylation is perhaps the most characterized and has been found associated with actively transcribed regions of chromatin. HATs are fundamental in many cellular processes such as replication, repair, cell cycle progression, differentiation, and apoptosis. Their functions in nuclear import, p53-mediated processes, and inflammatory responses have also been described [Kalkhoven, 2004]. Moreover, their role in gene activation is imperative in forming scaffolds that bridge basal transcriptional machinery with coactivators and chromatin remodeling complexes in a promoter-dependent and cell-specific context [Grunstein, 1997; Roth et al., 2001]. HATs modify a wide array of regulatory factors [Sterner and Berger, 2000] mediating protein–protein interactions, and themselves are targets of

phosphorylation by PKCδ, phosphorylated ERK1/2, PKA, and Cdk2 signifying their importance in the integration of signaling pathways [Kalkhoven, 2004].

With HAT function intimately linked with many cellular processes, it is not surprising that defects in their expression and activity play a causal relationship with diseases. In hematological malignancies such as acute myeloid leukemia, therapy-related meylodysplatic syndrome and mixed lineage leukemia, chromosomal translocations and inversions produce chimeric HATs that have gain of function enabling the fusion proteins to be mistargeted and acquire new interacting partners that expand their specificity and repertoire of protein and promoter targets [Iyer et al., 2004; Drobic et al., 2005]. Mutations, deletions, and loss of heterozygosity that lead to non-functional HATs have also been reported in primary tumors such as breast cancer, glioblastomas, gastric, colon, and pancreatic cancers [Kalkhoven, 2004]. The disorder Rubinstein–Taybi syndrome is known to result from mutations that create non-functional CBP [Ausio et al., 2003; Cho et al., 2004]. Further, mutated polyglutamine proteins cause CBP to be sequestered and subsequently inactivated, and this loss of CBP function is thought to characterize neurological disorders such as Huntington disease. Loss of function of CBP has also been observed in other diseases such as Alzheimer's disease, amytrophic lateral schlerosis, and spinal and bulbar muscular atrophy [Kalkhoven, 2004; Rouaux et al., 2004].

Although much is known about HATs in terms of their substrates, cellular function and biological implications in diseases, the development and clinical applications of HAT specific inhibitors continue to lag behind that of deacetylases inhibitors. To date, quite a few inhibitors have been identified such as lysyl-CoA, H3-CoA-20, and anacardic acid but their low cell permeability prevents their approved applicability in clinic [Drobic et al., 2005]. Recently, a naturally-occurring compound garcinol was found to be a potent, cell permeable HAT inhibitor although further systematic study of this molecule needs to be addressed [Varier et al., 2004].

## HISTONE DEACETYLASE INHIBITORS AS THERAPEUTIC MOLECULES

HDACs modify chromatin by removing acetyl groups from the amino-terminus of histones. HDACs are also responsible for removing acetyl groups from various other proteins including MyoD, p53, Hsp90, GATA-1, and tubulin [Juan et al., 2000; Hubbert et al., 2002; De Ruijter et al., 2003; Marks et al., 2003]. Three families of HDACs exist in mammals. Classes I and II are distinguished by homology to the yeast proteins Rpd3 and Hda1 respectively, while class III HDACs can be defined by a requirement for nicotinamide adenine dinucleotide. HDACs function dynamically with HATs to maintain a delicate balance of histone acetylation within the nucleus. Numerous studies have linked the acetylation of histones with transcriptional status.

HDACs are found in multi-protein complexes with transcription factors, tumor suppressors and oncogenes. Abnormal HDAC activity, recruited by altered protein partners, is a common theme in hematological cancers. In these cancers fusion proteins such as RAR-PML and RAR-PLZF could recruit HDACs through N-CoR and SMRT and cause aberrant transcriptional repression that prevents differentiation [Hong et al., 1997; De Ruijter et al., 2003].

Inhibitors of HDACs include natural and synthetic molecules. They fall into the following five classes: carboxylates, benzamides, cyclic peptides, electrophillic ketones, and small molecule hydroxamates. Potency varies with small molecule hydroxamates such as suberoylanilide hydroxamic acid (SAHA) and cyclic peptides inhibiting HDACs when present in nanomolar concentrations, while carboxylates like butyric acid require millimolar amounts [Secrist et al., 2003]. The structural classes appear diverse but contain several similar properties. Each contains a surface recognition domain, a linker domain and a metal binding domain [Miller et al., 2003]. Crystal structures of HDAC/HDAC inhibitor complexes show inhibitors blocking substrate access by binding to the catalytic site [Finnin et al., 1999].

Exposing transformed cells, whether cultured or in animal models, to HDAC inhibitors leads to differentiation, growth arrest, and apoptosis [Marks et al., 2001, 2003; Secrist et al., 2003]. Although both normal and tumor cells become enriched in acetylated histones, sensitivity to HDAC inhibitors is ten-fold higher in tumor cells [Richon et al., 1998; Scott et al., 2002; Kelly et al., 2003; Marks et al., 2003]. The mechanism by which HDAC inhibitors exert anti-cancer effects is not clear, but several

findings implicate chromatin structure in the process. Induction of histone acetylation should, at least in theory, result in a less condensed chromatin structure. Our current belief that HDACs are distributed throughout the genome would predict this decondensation to be widespread and affect the transcription of a large number of genes. In practice the result of treatment with HDAC inhibitors differs by cell type and has been shown to affect only a small percentage of the genome, having both inductive and repressive effects [Van Lint et al., 1996; Della et al., 2001; Butler et al., 2002; Suzuki et al., 2002; Dehm et al., 2004]. For example, a study of the transcriptional effects of SAHA on multiple myeloma cells found marked transcriptional changes in many genes related to pathology of the disease. Altered transcriptional status included the repression of anti-apoptotic genes and those involved in transformation and proliferation [Mitsiades et al., 2004]. Several sets of genes including insulin-like-growth-factor and its receptor and IL-6R and gp130 were repressed [Mitsiades et al., 2004]. SAHA treatment also prevented consensus-site binding of NF-κB and suppressed the oncogenes N-*ras* and raf-1, but transcriptional activation did not occur at genes known to be involved in differentiation [Mitsiades et al., 2004].

Changes to chromatin may indeed be the underlying cause of transcriptional activation by HDAC inhibitors. Structural changes in the promoter region of $p21^{WAF1}$, one of the genes most commonly upregulated after exposure to HDAC inhibitors, have been observed following treatment [Sambucetti et al., 1999; Richon et al., 2000; Gui et al., 2004]. The timing of these alterations, including increased acetylation of H3 and H4, correspond to an increase in DNAse 1 sensitivity and restriction enzyme accessibility, indicating that a less compact chromatin structure is involved [Gui et al., 2004]. Treatment also altered the assembly of proteins at the promoter. RNA polymerase II increased, while HDAC1 showed a decrease in association [Gui et al., 2004]. DNAse 1 studies at other genes not induced by HDAC inhibitor treatment showed no increase in accessibility, indicating that this decondensation of chromatin is not genome-wide [Gui et al., 2004]. However, HDAC inhibitors may alter gene expression at levels other than transcription. For example, in HepG2 cells, HDAC inhibitors, butyrate and trichostatin A increased $p21^{WAF1}$ expression not by enhancing the activity of the promoter but by stabilizing $p21^{WAF1}$ mRNA [Hirsch and Bonham, 2004]. It is clear that HDAC inhibitors can alter gene expression programs at multiple levels other than altering the status of acetylated proteins (histones, transcription factors, chromatin structural proteins, and chromatin remodeling enzymes) [Davie, 2003a]. To support this view, treatment of JB6 epidermal cells with trichostatin A resulted in the rapid activation of ERK and p38 through stimulation of the Ras-MAPK and stress kinase pathways, resulting in the activation of MSK1/2 and phosphorylation of S28 of H3 [Zhong et al., 2003].

Another study, conducted by Marchion et al., found an interesting connection between HDAC inhibitors and potential DNA damage by topoisomerase II inhibitors [Marchion et al., 2004]. Topoisomerase II inhibitors are commonly part of adjuvant breast cancer therapy and result in DNA damage by stabilizing the DNA–topoisomerase II complex. Prior treatment of cells with SAHA leads to a decondensation of chromatin and an increase in binding of the topoisomerase II inhibitor. Thus SAHA effectually potentiates DNA damage by topoisomerase II inhibitors [Marchion et al., 2004].

These findings indicate that treatment with HDAC inhibitors results in the formation of regions of altered chromatin structure that lead to increased susceptibility, whether it be to transcription factors, the RNA polymerase complex, or topoisomerases. Selection of regions for decondensation likely depends on the balance of chromatin modifiers/remodelers recruited to the area and may be influenced by the cellular environment.

## HISTONE METHYLATION AND CANCER

The four core histones are modified by methylation of lysines and arginines located in the N-terminal tail and histone fold domains (Fig. 1). Histone methylation is catalyzed by histone methyltransferases, which are a large family of enzymes that have specificity for a histone, the modification site (lysine or arginine), and chromatin region [Davie, 2004]. H3 methylated at K4 and K79 is located in transcribed regions of the genome, while H3 methylated at K9 and H4 methylated at K20 are present in heterochromatin regions, the histones of which are hypoacetylated [Liang et al., 2004; Schotta et al., 2004; Schubeler et al.,

2004]. Recent studies demonstrate that histone methyl arginines can be removed by the action of human peptidylarginine deiminase 4 (PAD4), which converts methyl R to citrulline and the release of methylamine [Cuthbert et al., 2004; Wang et al., 2004]. It is unclear whether histone methylation at lysines is also reversible. However, histone exchange occurring during transcription is one mechanism by which the core histones are dislodged from the transcribed DNA and replaced by a histone that is not methylated [Workman and Abmayr, 2004].

SMYD3 (SET- and MYND-domain containing protein 3) is an H3 K4 histone methyltransferase and sequence-specific DNA binding protein that is overexpressed in colorectal carcinomas and hepatocellular carcinomas. Suppression of SMYD3 expression inhibited the growth of colorectal carcinoma and hepatocellular carcinoma cells. SMYD3 is involved in the activation of oncogenes and genes associated with cell-cycle regulation [Hamamoto et al., 2004]. EZH2 is a H3 K27 histone methyltransferase that is a component of the embryonic ectoderm development (EED)–EZH2 complex. This histone methyltransferase is overexpressed in prostate and breast cancer cells. Pho and Pho1 are sequence-specific DNA binding proteins that bind to the Polycomb response element and recruit the EED–EZH2 complex. Methylated H3 K27 in turn recruits Polycomb group (PcG) proteins and the Polycomb repressive complex 1 to silence specific genes. PcG proteins maintain the silenced state of homeotic genes. Thus, deregulation of EZH2 may result in alteration of chromatin structure and deregulation of the downstream targets of the EED–EZH2 complex [Cao and Zhang, 2004].

Alterations in the levels and distribution of methylated histones in cancer cells have been reported. Neutrophil granulocytes from healthy individuals lacked H3 K9 methylated isoforms, while granulocytes from patients with chronic myeloid leukemia had H3 mono- and dimethylated K9 [Lukasova et al., 2005]. Using an antibody recognizing methylated lysines independent of their lysine position in the histone, leukemic T-cell Jurkat cells had methylated histones located to numerous distinct clusters. This was in contrast to the homogeneous distribution of chromatin with methylated histones in normal G0 lymphocytes. Also, the chromatin with lysine methylated histones was concentrated more peripherally in colon carcinoma compared to nuclei of normal colon epithelial cells [Cremer et al., 2004].

## HISTONE METHYLATION, HETEROCHROMATIN PROTEIN 1 (HP1), AND BREAST CANCER

Methylated K9 H3 is localized primarily to heterochromatin regions of mammalian cells [Dillon and Festenstein, 2002]. H3 methyl K9 binds avidly to the chromodomain of HP1, recruiting the protein to heterochromatic regions [Bannister et al., 2001; Jacobs et al., 2001; Lachner et al., 2001; Nakayama et al., 2001]. HP1 will not bind H3 methyl K4 [Bannister et al., 2001]. Further, HP1 interacts with the H3 K9 methyltransferase SUVAR39H1. Thus, models have been proposed in which HP1 recruited by a nucleosome bearing an H3 methyl K9 will enable the HP1 bound H3 K9 methyltransferase to methylate the neighboring nucleosome; hence, a self-propagating mechanism for the spreading of heterochromatin occurs [Bannister et al., 2001].

In addition to heterochromatic silencing, SUV39H1 H3 methyltransferase and HP1 are involved in repression of euchromatic genes. The transcription factor E2F has a pivotal role in regulating the expression of S-phase-specific genes. Repression of these genes is through the retinoblastoma (Rb) protein, which binds to E2F. Rb recruits histone methyltransferases and HDACs to repress gene expression [Luo et al., 1998; Nielsen et al., 2001; Vandel et al., 2001]. Rb bound to E2F recruits SUV39H1 to the S-phase-specific gene promoter (e.g., cyclin E), which in turn recruits HP1 [Nielsen et al., 2001; Vandel et al., 2001]. Disruption of SUV39 in mouse embryonic fibroblasts increased the expression of cyclin E. Phosphorylation of Rb abolishes its association with HDAC and H3 K9 methyltransferase.

Human cells express three forms of HP1 proteins. $HP1^{Hs\alpha}$ and $HP1^{Hs\beta}$ are present in pericentric heterochromatin and to a limited extent with euchromatin, to which $HP1^{Hs\gamma}$ primarily localizes. Breast cancer cells with a metastatic phenotype have low levels of $HP1^{Hs\alpha}$. The invasive properties of these cells were attenuated when $HP1^{Hs\alpha}$ levels were restored. In this situation the epigenetic program was compromised by the down-regulation of a protein key in silencing [Kirschmann et al., 2000; Li et al., 2002].

## DNA METHYLATION AND HISTONE METHYLATION

DNA methylation is a prominent epigenetic process involved in gene silencing, with deregulation of this process often being observed in cancer cells [Verma and Srivastava, 2002]. In normal mammalian cells, CpG islands in the regulatory regions of genes are not methylated, while CpG residues that are not clustered are usually methylated by the DNA methyltransferase (DNMT1). In cancer cells global DNA hypomethylation and region specific hypermethylation occurs [Robertson and Jones, 2000]. Both DNMT1 and DNMT3b maintain the aberrant methylation DNA hypomethylation may result in the activation of normally silent genes, which when activated contribute to tumorigenesis and metastasis. Hypermethylation of the CpG islands in regulatory regions of genes has the opposite affect of silencing the expression of genes involved in the prevention of cancer, for example, tumor suppressor genes [Brown and Strathdee, 2002]. The cyclin-dependent kinase inhibitor, p16, is silenced in many types of cancer by hypermethylation of a CpG island residing in its promoter [Kondo et al., 2003]. The loss of p16 expression results in loss of cell cycle regulation, resulting in a growth advantage to affected cells.

There is a dynamic relationship between histone modifications, chromatin structure, and DNA methylation [Szyf et al., 2004; Ting et al., 2004]. Histone acetylation and gene activation, results in DNA demethylation [Szyf et al., 2004]. Conversely, inactivation of genes leads to a chromatin with a low steady state level of histone acetylation and H3 K9 methylation, resulting in the recruitment of DNMT1 and DNA methylation of regulatory regions. Investigations following the order of events ensuing from gene inactivation demonstrate that a low level of DNA methylation at the promoter recruits the methylated DNA-binding protein MBD2, which recruits HDACs and DNMT1. The HDACs deacetylate the histone, for example, acetyl K9 and acetyl K4 of H3. DNMT1 recruitment results in subsequent methylation of the promoter, leading to the recruitment of the methyl DNA-binding protein MeCP2. MeCP2 recruits an H3 K9 methyltransferase, resulting in K9 H3 methylation [Stirzaker et al., 2004]. This program of events may be reversed when cancer cells are treated with the DNA demethylating drug 5-Aza-deoxy-cytidine (5-Aza-dC) [Szyf et al., 2004], with DNA demethylation of the promoter resulting in the loss of methyl H3 K9 and acetylation of H3 and methylation of H3 K4 after the resumption of transcription [Fahrner et al., 2002]. A recent study comparing the effects of trichostatin A, an HDAC inhibitor, to that of 5-Aza-dC on gene expression (activation or repression) in HCT116 colon cancer cells, revealed that responses to either agent were very similar. These observations suggest that 5-Aza-dC and trichostatin A have a common upstream pathway to influence gene expression [Gius et al., 2004].

## FUTURE DIRECTIONS

There is still much to be learned about the impact of mutations in oncogenes and tumor suppressor genes on epigenetic processes. Further appreciation of the enzymes involved in the epigenetic pathways and the regulation of their activities will provide new opportunities for cancer therapy. There is considerable interest in the inhibitors of HDACs and DNA methylation in the treatment of cancer. However, there is the concern that these inhibitors may activate genes involved in tumorigenesis by stimulation of Ras-MAPK signaling pathways or demethylating regions resulting in the derepression of genes involved in tumorigenesis and/or metastasis [Zhong et al., 2003; Szyf et al., 2004]. The list of all possible histone modifications is not yet complete. With the implementation of mass spectrometry approaches, novel histone modifications are being found. A particularly interesting class of modifications is those that affect the interaction between the histone fold and nucleosomal DNA. It is postulated that modification of these amino acids by acetylation, methylation, or phosphorylation will aid in nucleosome mobility and nucleosome dynamics [Cosgrove et al., 2004]. Hence these modifications in the histone fold may have key roles in the more open chromatin structure often observed in cancer cells. In addition to identifying new therapies in the treatment of cancer, there is considerable interest in how diet and environmental factors influence epigenetic programs. It is in this area that great gains can be made in reducing the burden of cancer on our health care systems.

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